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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 9/50, 9/16

A2

(11) International Publication Number: WO 96/18388

(43) International Publication Date: 20 June 1996 (20.06.96)

(21) International Application Number: PCT/GB95/02925

(22) International Filing Date: 14 December 1995 (14.12.95)

(30) Priority Data:
94309417.7 16 December 1994 (16.12.94) EP
(34) Countries for which the regional or international application was filed: GB et al.

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(81) Designated States: AL, AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: CROSS-LINKED MICROPARTICLES AND THEIR USE AS THERAPEUTIC VEHICLES

(57) Abstract

A sterile powder comprising microparticles, 0.1 to 50 μm in diameter, obtainable by spray-drying and cross-linking a water-soluble material having free functional groups, is characterised in that the microparticles are hydrophilic, can be reconstituted in water to give a monodisperse suspension, and have retained said groups available for derivatisation. The particles are linked to drugs or other functional molecules, and used as vehicles in therapy.

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CROSS-LINKED MICROPARTICLES AND THEIR USE AS THERAPEUTIC VEHICLES

Field of the Invention

This invention relates to cross-linked microparticles and their use as therapeutic vehicles.

Background of the Invention

Microparticulate carrier systems are increasingly attracting attention for use in the parenteral delivery of therapeutic and diagnostics agents. A plethora of microparticle technology systems and chemistries has been proffered as vehicles to deliver agents subcutaneously, intravenously and intra-arterially. There are several key aspects to the "ideal vehicle". These include size, size distribution, payload, rate of biodegradation, ease of use, release kinetics and scalable reproducible production. Individual aspects of this "ideal vehicle" have been successfully addressed by others, notably drug payload, rate of biodegradation and, in part, size and size distribution.

Known vehicles have been manufactured by various techniques, largely solvent and emulsion-based. A disadvantage of these methods is that control of the key elements of the vehicle was attempted within one or two steps of the production. Thus, size, size distribution, payload and rate of biodegradation were all imparted on the product in a single, dynamic environment, typified by single and double emulsion systems or solvent evaporation techniques. Typically, for emulsion processes, the solution of drug, polymer and surface-modifying agents has been mixed with an insoluble solvent, emulsified, heated or stabilised to fix the particles, and then cleansed to remove oils or solvent incompatible with parenteral use.

The reaction vessel in emulsion or solvent evaporation systems is a principal characteristic of the prior art techniques. Within this vessel, the control of the microparticle morphology is achieved by balancing the interfacial forces of oil and water components, the

interaction of solute at the interface, the balance between agitation, heat and shell formation and, of course, the incorporation of active within the polymer matrix. However, such technologies are largely incompatible with large-scale pharmaceutical manufacture required for a parenteral agent.

Almost without exception, the control of size and size distribution of known microparticles was vastly inferior to the size control attained by the spray-drying techniques described in the PCT publications WO-A-9112823, WO-A-9218164 and WO-A-9408627, in the production of microparticles for use in echo-contrast imaging and other potential parenteral uses. The acute toxicity of intravenous microparticles is largely associated with capillary blockage in the pulmonary circulation, concurrent decrease in the pulmonary venous pressure and loss of compliance. The relationship between particle size and LD toxicity is well recorded. Our own data show the precipitous elevation in toxicity of iv particles, with a mean size in excess of 6 µm, the notional capillary size in lung tissue for non-deformable microcapsules.

Typically, the larger the mean size of the capsules, the significantly broader their size distribution, and the range of microparticle sizes can span two orders of magnitude. For therapeutic use, such as chemo-embolisation, the prospect of injecting a microparticle preparation containing particles ranging in size from 5-100 µm is largely inconsistent with the concept of highly regionalised targeted delivery. At the upper end, there is the prospect of embolising major vessels up to and above 100 µm in diameter, with the attendant risk of necrosing large perfusion territories; at the smaller end of the range, what essentially amounts to systemic distribution becomes possible.

The mechanism by which sustained release has previously been most commonly achieved in microparticle systems has been the control of matrix erosion and release

to the surrounding medium of embedded or imbibed actives. The actives have either been incorporated at the time of particle production or imbibed into the matrix following fixation or stabilisation.

The incorporation of drugs into the matrix of the known microcapsules required heating in the presence of water and, inevitably, oxygen. This would almost certainly lead to adulteration of the drug by oxidative damage or uncontrolled cross-linking to the vehicle. In those cases where chemical stabilisation is used, the potential loss of active would be even worse.

Another mechanism of slowing or modifying release rates of drugs from soluble polymeric carriers has been to link the actives *via* covalent linkages to the soluble polymer. In general, this has not been applied to microparticulate systems where drugs, ligands or antibodies are linked to particulate carriers.

The main impediment to linking actives to prior art microparticles, is the latter's relative hydrophobicity. Since many of the chemical reactions required to achieve linkage are carried out in aqueous medium, such hydrophobic microparticles are almost impossible to derivatise. Where previous workers have produced hydrophilic microcapsules, they required complex formation in the presence of hydrophilic polymer, in an emulsion process.

The rate of biodegradation of microcapsules is determined largely by the extent of cross-linking. In the prior art systems, changes in cross-linking have detrimental effects upon drug loading and the ability subsequently to formulate the microparticles. Little effort was expended in attempting to manipulate this parameter to control the rate of biodegradation and drug release.

Microparticles of the prior art have required significant amounts of surfactants or sonication to achieve monodispersed suspension in aqueous media. Even when reconstituted, the microparticles have a propensity to

agglomerate and are thus difficult to administer through hypodermic syringes.

Summary of the Invention

This invention is based on the discovery that microparticles of the type described in the PCT publications WO-A-9112823, WO-A-9218164 and WO-A-9408627, having good particle characteristics, can retain, even after thermal cross-linking, their hydrophilic properties and the ability to be reconstituted in water to give a monodisperse suspension. Further, functional groups such as carboxyl, amine, hydroxyl or sulfhydryl groups in the starting material are retained, and are available for derivatisation.

According to the present invention, a sterile powder comprises smooth, spherical microparticles, 0.1 to 50 μm in diameter, of cross-linked materials, the microparticles being hydrophilic and capable of reconstitution in water to give a mono-disperse suspension, and which additionally comprises a physiologically or diagnostically-active component linked directly or indirectly to microparticles *via* free functional, e.g. amine, hydroxyl, carboxyl or sulfhydryl, groups thereon.

Description of the Invention

This invention preferably utilises a microparticle production technique of the type described in the PCT publications, *supra* (the contents of which are incorporated herein by reference), in which there is tight control over size, size distribution, payload, rate of biodegradation and release kinetics, providing ease of use and scalable production. The microparticles of this invention may be tailored at will to suit the application whilst retaining, in all cases, the ability to produce the loaded vehicle at scale to high levels of pharmaceutical practice and always with the same level of control. In addition to control of size, independent control, in individual steps, is possible for size distribution; rate of fixation or, reciprocally, rate of biodegradation; drug loading; and formulation and

finishing. As previously disclosed in the PCT publications, *supra*, the Applicants have a fully scaled process which can produce microparticles of the nature specified. The process may be operated to pharmaceutical standards without the ingress of foreign particulates that would most certainly preclude parenteral use of microparticles produced by many of the prior art processes.

The present invention relates to the production of microparticle preparations for intravenous, intraarterial and *ex vivo* use. Intravenous particle suspensions, on reconstitution in diluent, preferably contain less than 5% by volume of particles larger than 6 μm. Furthermore, the size distribution is near Gaussian in shape, with some 50% of particles lying within a range of 5 μm, preferably 3 μm, more preferably 2 μm and most preferably 1.2 μm. A desirable distribution has 80% of particles in a range of 3 μm. (All distributions quoted on a volume or mass basis). One preferred embodiment of the invention is powders wherein 95% of the particles are smaller than 6 μm, and 80% of the particles are in the range of 1 to 6 μm, especially for iv administration. Another preferred embodiment is powders wherein 90% of the particles are smaller than 20 μm, and less than 5% by volume are smaller than 6 μm, especially for intraarterial administration.

For larger particle systems, by utilising a combination of highly controlled spray drying and a subsequent fractionation step, it is possible to produce microparticles of sufficient size and tight size distribution such that, following intraarterial administration, systemic release is eliminated and only vessels smaller than 20 μm become embolised.

In one embodiment of the current invention, we have incorporated active within the feedstock for spray drying and subsequently stabilised the particle. The advantage we have gained is vastly superior control of morphology and payload over previous methodologies.

The process used in this invention may involve the deposition of wall material and drug into dry powder state, with negligible water content, capable of being reconstituted to the original soluble components.

In this invention, cross-linking can be used to control the release rates of actives. For example, in one embodiment, the extent of cross-linking, and hence the rate of degradation, is "set" prior to attachment of the active. The active ligand may then be attached, and shows a controlled release profile determined by the extent to which the matrix was cross-linked. This has the advantage that a steady rate of release is observed without the well-known "burst" effect.

A further aspect of this invention is the manipulation of cross linking. Potential levels of derivatisable groups and temperature/time required for cross-linking may be controlled by the incorporation of additives which alter these parameters. Inclusion of lysine or polylysine, glutamate or polyglutamate, phenylalanine and tyrosine into the feedstock can have the effect of increasing or decreasing the biodegradation rate of the final microparticle preparation. Additionally, the incorporation of these additives can significantly increase the number of potential groups to which actives, or ligands, may be attached. Furthermore, incorporation of these additives can reduce the time/temperature at which microparticle stabilisation occurs during heating of the soluble microparticles formed after spray drying.

Accordingly, therefore, while the present invention provides microparticles that are especially suitable for delivery to defined sites, owing to their narrow particle size distribution, a combination of desirable features has been found that make the microparticles especially useful.

It has been discovered that microcapsules manufactured using the techniques described, have several superior properties over the prior art microparticles with regard to the ability to handle, derivatise and formulate the

microparticles. Thus, for example, the Applicants' technology enables the production of microparticles which apparently retain a substantial degree of secondary structure, and thus hydrophilicity instead of being denatured, and rendered insoluble, with hidden functional groups, as in the prior art. In particular, the microparticles:

(i) possess significant levels of amine, hydroxyl or carboxyl groups, or a combination thereof, for derivatisation,

(ii) are highly hydrophilic with full access by aqueous media for the derivatisation of the active groups,

(iii) may be manipulated in dry or wet state to yield dry final formulations that do not require surfactants or sonication to yield monodispersed suspensions of particles,

(iv) biodegrade and release active principles at a rate determined by the composition and cross-linking of the shell,

(v) may possess sizes ranging from 0.01 μm to 100 μm, and

(vi) are retained in circulation <u>in vivo</u> for periods of time (60 min or more), considerably longer than for the more hydrophobic known microparticles, and longer than for liposomes; opsonisation is reduced, and thrombogenic potential minimised.

The particle size is preferably below 4 μm for intravenous administration, and between 8 and 30 μm for intraarterial administration. Especially for larger particles, fractionation is an optional extra step. This particle size range can be expressed such that the ratio of the interquartile range to mean diameter is 0.2 to 0.5.

The microparticles of this invention may be derivatised by conjugation of drugs, ligands, peptides or proteins directly to the carrier using the carboxyl or

amine groups of the basic capsules or additives made to the feedstock for spray drying. For example, conjugation may be achieved using glutaraldehyde, EDCI, terephthaloyl chloride, cyanogen bromide or reductive amination. Alternatively the ligand, drug, protein or peptide may be linked *via* a biodegradable hydroxy acid linker of the kind disclosed in WO-A-9317713 (Rijksuniversiteit Groningen), the content of which is incorporated by reference.

A further advantage of this invention lies in the ability to formulate and present the product as a dry sterile powder.

Microcapsules of this invention in powder form do not have an absolute requirement for surfactants to ensure a monodispersed suspension on reconstitution. Once reconstituted, they do not agglomerate, and can be administered *via* syringe.

An aspect of the present invention is a water-compatible system manufactured from biocompatible materials. It could not be anticipated that the microparticles of the current invention could be insolubilised by heating yet retain sufficient secondary structure to remain highly hydrophilic. Evidence of the retention of secondary structure is obtained by examination of the particle isoelectric point (PI) which, at pH 4.5 to 5, is very similar to native albumin. Normally, full denaturation of albumin leads to a significant rise in PI, to a value of 6.5 to 7.0.

Further, digestion of protein microparticles with protease yields peptides which, when compared with digests of the starting soluble protein, show near identical profiles, by HPLC analogues. In addition, acid hydrolysis of protein microparticles and protein starting material show stikingly similar amino-acid content. These two analyses support the observation that the protein in the microparticles is largely native.

The novel particles are hydrophilic and have the potential to circulate for periods in excess of one hour,

Table 1

Spray Dryer Condition	Setting
Inlet Temperature	220°C
Outlet Temperature-Initial	85.2°C
Outlet Temperature-Final	84.0°C
Atomisation Pressure	7.5 bar
Damper Setting	0.5
Feed Rate	3.88 g/min
Stock Solution	25% ethanol 100 mg/ml HSA

The spray-drying process produced 17.21 g of microcapsules. Microcapsules from this single production batch were divided into equal sized aliquots and heat-fixed at 175°C for 45, 55 and 75 minutes respectively. The heat fixation process renders the soluble microcapsules from the spray-drying process insoluble, by cross-linking some of the amino acids within the albumin structure. The three different heat-fixed microcapsules were sized in an aqueous system using a Multisizer II (Coulter Electronics). The microcapsules had a mean size of 3.28 ± 0.6 μm and with 90% of the mass within 2-5 μm.

Prior to binding any active component to the microcapsules, those heat-fixed for 55 minutes were analysed in various ways for their suitability as drug carriers.

Free Thiol Analysis

The free thiol group present in the albumin molecule is very susceptible to modification and hence it can be used as a measure of the state and condition of the albumin. Similarly, it should be present within the microcapsule structure providing the albumin molecule was not disrupted during formation.

Analysis of the free thiol group was carried out reacting the albumin microcapsules with DTNB, i.e. 5,5'-dithiobis(2-nitrobenzoic acid). If the free thiol is present, it reacts with the DTNB to yield a nitrobenzoic acid derivative that absorbs at 412 nm. The absorbance of

a 12 mg/ml suspension of microcapsules at 412 nm was measured. To the suspension, 50 μl of a 20% solution of DTNB in TRIS buffer was added, incubated for 10 min at room temperature and the absorbance measured. The difference between the two absorbances was calculated and from the molecular extinction coefficient of the reaction product, the concentration of the free thiol present in the microcapsules was calculated. The molecular ratio of thiol groups measured in the microcapsules was 0.4785. This compared to a value of 0.5045 for native albumin. This was not a significant difference and it was concluded that the free thiol group was unchanged during microcapsule manufacture.

The microcapsules (both soluble and insoluble) and native albumin were broken down to their constituent amino-acids by vapour phase hydrolysis using concentrated HCl at 120°C for 24 hr. The samples were then derivatised by the addition of triethylamine in 50% ethanol and followed by triethylamine and PITC in ethanol. The derivatised samples were analysed by HPLC, and the amino-acids detected at a wavelength of 254 nm.

Table 4 (at the end of the description) shows the resultant amino-acid compositions. Unexpectedly there is no significant difference between the various samples, with only very small losses of amino-acids containing carboxyl, hydroxyl and amide groups after insolubilisation of the microcapsules.

Peptide Analysis

Pepsin digestion of the microcapsules and albumin was performed using a 1 ml acidified solution of microcapsules or albumin to which was added 20 μl of a 1% pepsin solution. Digestion was carried out at 37°C for 24 hr followed by a second addition of pepsin and a further incubation at 37°C until the samples were completely digested. HPLC analysis of the resultant lysates was carried out using an acetonitrile gradient in 0.1% TFA, measuring absorbance at 214 nm.

Trypsin digestion of the microcapsules and albumin was performed on samples initially treated with guanidine-HCl, DTT and iodoacetamide, to open the protein structure. 0.2% trypsin was added to these pre-treated samples and incubated at 37°C until completely digested (additional trypsin was added if required). HPLC analysis of the lysates was carried as detailed above.

The HPLC analyses showed no significant differences between the microcapsule and albumin structures. This confirms that there is significant retention of secondary and tertiary protein structure after microcapsule insolubilisation.

Coupling to FITC

FITC (fluorescein isothiocyanate) binds covalently to amino groups on the microcapsules and exemplifies the principle of derivatising charged groups, namely lysine residues, with drugs which are subsequently released by degradation of the microcapsule matrix itself.

FITC was covalently bound to three microcapsule batches. A ratio of microcapsules to FITC of 15:1 was used. 12.5 mg FITC was added to the suspended microcapsules and the mixture was incubated at 30°C for 30 minutes. Excess fluorescein was removed by washing the microcapsules until no fluorescein was present in the washing, i.e. no leaching of the marker was observed.

The microcapsules were digested with Proteinase K at a concentration of 0.4 EU/ml. The fluorescein was released from the microcapsules as they were digested, and was measured by sampling the microcapsules suspension at various time intervals. The released FITC was separated from the microcapsules by centrifugation and quantified by measuring the absorbance at 493 nm.

The results showed that, the less heat-fixed the microcapsules, the more rapid the initial release of fluorescein. However, after 225 minutes, all samples had released greater than 90% of the fluorescein. The amount of FITC bound to the different heat-fixed microcapsules was

similar, with approximately 10 ± 0.5% mole/mole loading for all three batches.

Release rates for bound actives can thus be adjusted by "setting" the degradation rate of the microcapsules prior to the attachment of the actives.

Microcapsules of Example 1 were incubated with whole human blood for 30 minutes at 37°C to determine if the microcapsules were able to stimulate platelet activation. The concentration of microparticles was equivalent to a dose of 2000 x 10^6 particles/kg. After 30 minutes incubation, the serum was tested for effects on platelet aggregation stimulated with collagen ADP and arachidonic acid. Effects on general hemostatic mechanisms were assessed by measurement of procoagulant activity, partial thromboplastin time; prothrombin time by appearance of fragments 1+2 and fibrinopeptide A; and fibrinolytic activity by examination of euglobulin lysis time.

At this concentration, there was no evidence of any untoward effects upon the assays tested. Thus the results suggest that the microparticles are inert and hydrophilic, unlike microparticles made by an emulsion processes.

In a further test, microparticles manufactured by the method of Example 1 were sterilised by gamma irradiation by exposure to a Co^{60} source and received a dose of 25-35 Kgray. The microcapsules were reconstituted in aqueous diluent at a concentration of 1.5 x 10^9 microparticles/ml and administered to healthy male volunteers at doses ranging from 25-300 x 10^6 microparticles/kg under ethical committee approval. The microparticles were hollow and contained air which enabled their passage and persistence in the blood stream to be followed using ultrasound imaging.

Using an Acuson-128, grey scale 2D images of the right and left ventricle were acquired to monitor the circulatory life time following IV dosing. For opacification of both the left and right ventricle to occur, significant levels of microparticles must be present in the chambers. At

doses from 25 x 10^6/kg upwards, the opacification in the right and left ventricles persisted for a period of 1 hour or more, showing that significant quantities of particles remained in the circulation.

This data shows the basic microcapsule vehicle to be inert to the coaggulatory machinery in the blood, and hence ideally suited to carry therapeutics. This is completely contrary to microparticles made by emulsion processes which show rapid RES uptake and circulatory half-lives of 10 minutes or less. Furthermore, the microparticles do not require derivatisation with co-block polymers to enhance the circulatory half-life.

Example 2

This Example shows that additives can be included in the spray-drying feedstock of the microcapsule wall-forming material, such that the resultant microcapsules can be heat-fixed at a lower temperature. Additives which allow lower cross-linking (insolubilisation) temperatures of the microcapsule polymer have utility when active drugs are co-spray-dried and hence incorporated in the matrix. By using these additives, microcapsules with heat-sensitive actives can be insolubilised at advantageous lower temperatures.

To the spray-drying feedstock, 5 mg/ml tyrosine was added, and microcapsules were formed, using the method detailed in Example 1. No changes in the spray-drying conditions were required to obtain microcapsules.

The collected microcapsules were heat-fixed as before, but at a temperature of 100°C for 55 minutes, significantly lower than the normal 175°C for 55 minutes, to achieve the same cross-linking. The microcapsules produced had a mean size of 3.28 ± 0.6 μm with 90% of the mass within 2-5 μm.

Example 3

Example 1 details the production of 3 μm microcapsules. This Example shows that, by adjustment of the spray-drying conditions and the use of a secondary stage classification processing step, larger microcapsules

may be produced with excellent control over size and size distribution.

20% HSA was spray-dried under the conditions shown in Table 2. The collected microcapsules were heat-fixed at 175°C for 55 minutes, deagglomerated and then classified using an elbow jet classifier (see Table 3).

Table 2

Spray Dryer Condition	Setting
Inlet Temperature	220°C
Outlet Temperature-Initial	89.1°C
Outlet Temperature-Final	89.2°C
Atomisation Pressure	2.0 bar
Damper Setting	0.5
Feed Rate	20.1 g/min
Stock Solution	20% HSA

Table 3

Classification Conditions	Settings
Primary Air	0.6 barg
Secondary Air	2.0 barg
Venturi Air	8.0 barg

The middle classified fraction was collected and reformulated, as the classification process removes much of the excipient. The resultant free-flowing dry powder was characterised as before. The microcapsules had a mean size of 12 μm, with virtually no microcapsules below 6 μm, and 85% of the mass between 9-18 μm.

By removing particles smaller than 6 μm, systemic circulation of microcapsules, following intraarterial administration, is prevented due to capillary trapping. This has the advantage of localising the deposited drug, thereby reducing the overall amount of drug required to achieve therapeutic activity at the desired site. This is desirable, particularly in the case of cytotoxics since

systemic toxicity is the major cause of detrimental side-effects.

Antibodies were then bound to the microcapsule wall surface. A FITC IgG was used to aid the detection of the bound antibody.

To 5 mg of FITC-IgG, 35 mg of sodium periodate was added. The mixture was incubated at room temperature for 1 hour, after which 20 mg microcapsules was added. The suspension was stirred for 10 minutes and then the activated antibody was bound to the microcapsules by the addition of 30 mg sodium borohydride. The reaction was allowed to proceed for 2 hours at room temperature, after which time the microcapsules were collected and washed.

A sample of the microcapsules was reduced, releasing the light chains of the bound antibodies. The microcapsules were removed and the resultant filtrate collected. The presence of FITC-labelled antibody light chains in the filtrate was measured by the use of a fluorimeter.

The linkage of antibodies to the microcapsules may also be achieved by means of tri and tetrapeptide spacers. The peptides are covalently linked to the activated sugar ring on the antibodies using the periodate and borohydride reaction detailed above. The antibodies are then linked to the microcapsules *via* this peptide spacer using EDCI, as detailed in Example 6.

Example 4

This Example shows that the incorporation of additives into the spray-drying feedstock, for example HSA, will alter the chemical properties of the microcapsules produced as in Example 1, such that the number of chemical linkage sites may be greatly enhanced.

Poly-lysine was incorporated into the spray-drying feedstock at a concentration of 5 mg/ml. The spray-drying procedure was carried out as detailed in Example 1. The microcapsules produced from this modified stock had a mean

size of 3.5 ± 0.3 μm with similar physical characteristics to the HSA microcapsules such as resuspension properties.

FITC was bound to the poly-lysine microcapsules as detailed in Example 1. The microcapsules were washed until no further release of the FITC was observed. The FITC was retained bound to the HSA microcapsules, showing no release in aqueous suspension. The microcapsules were digested as detailed in Example 1 and the total amount of fluorescein bound to the microcapsules measured.

The release rate of fluorescein upon digestion showed a more rapid release than the standard microcapsules. However, a total of 20 molar % fluorescein was measured bound to the microcapsules, an increase of 50% over the standard microcapsule composition.

Example 5

This Example shows that actives may be linked to the shell and their subsequent release rate governed by degradation of the microcapsule itself.

To a 10 mg/ml solution of methotrexate, 25 mg carbodiimide (EDCI) was added. The solution was stirred for 4 hours to initiate and ensure complete activation of the methotrexate. 50 mg HSA microcapsules, produced as in Example 1, were added to the activated drug and stirred for 3 hours at room temperature. The methotrexate was chemically bound to the microcapsules *via* the amine groups on the albumin. The microcapsules were collected and washed to remove any loosely-bound methotrexate.

The methotrexate-linked microcapsules were characterised and also analysed for drug content. The microcapsules had a mean size of 3.2 ± 0.6 μm with 90% by mass between 2-5 μm. The analysis of the drug content showed that the microcapsules did not release the drug when resuspended in an aqueous medium. Moreover, a three month stability trial showed no drug release after this time. Proteinase K digestion of the albumin microcapsules released the bound drug which was shown to be linked to only a limited number of amino acids and small peptides.

Example 6

Doxorubicin was conjugate to microcapsules produced using the general method described in Example 1, with carbodiimide. 3 mg doxorubicin, 6 mg EDCI and 100 mg microcapsules were suspended in distilled water at pH 6.6 and 37°C for 20 hr with continuous stirring and resuspension and formulated.

The doxorubicin-microcapsules were analysed for drug content and characterised. The results showed no change had occurred to the characteristics of the microcapsules during drug conjugation. Drug loading was assessed on an enzyme-digested sample of microcapsules by HPLC analysis. A loading of 2% was measured and the drug was shown to be linked only to a limited number of amino-acids or small peptides.

It has been shown previously that the activity of doxorubicin bound to polymeric carriers proves beneficial in tumours showing the multidrug resistant phenotype.

Example 7

Microcapsules produced using the method of Example 1 were derivatised to carry 2-fluoro-5-deoxyuridine (FUdR). The drug was activated with succinic anhydride.

40 mg FUdR was added to 0.2M phosphate buffer at pH 8.3. To this solution 200 mg succinic anhydride was added and the reaction mixture stirred at 30°C for 2 hours, whilst maintaining the pH at 8.3 using IM NaOH. The pH of the reaction mixture was adjusted to 6.5, and 0.5 g microcapsules added. After stirring the suspension for 10 minutes, EDCI and N-hydroxysuccinimide in a ratio of 15:1 were added, and the coupling reaction allowed to proceed at room temperature. After 24 hours, the microcapsules were collected and washed, and the presence of FUdR on the microcapsules confirmed by HPLC analysis. The acid hydrolysis of the drug-linked microcapsules was carried out in an ASTED system using 1% TFA, and the subsequent release of the FUdR was monitored at 269 nm. 15% w/w FUdR was

found to be bound to the microcapsules *via* the acid hydrolysable linkage.

Cytotoxic Activity

Cytotoxic activity of the drug-microcapsule conjugates of Examples 5 to 7 was measured *in vitro*. The HSN cell line was used. This is a chemically-induced rat colonic sarcoma, which produces liver tumours in an animal model with a vascular pattern similar to that seen in human colorectal liver metastases. Cell kill was measured indirectly using MTT. This is reduced in active mitochondria to formazan, a coloured metabolite. HSN cells were incubated in multi-well plates to which was added a control dose of drug or drug-microcapsule lysate. After a range of exposure times, MTT was added to the wells and the concentration of formazan measured as an indication of cell death. The assay was calibrated for this cell line against a range of known cell numbers.

All three drugs (methotrexate, 5-FUdR and doxorubicin) had similar dose response curves for the native drugs and the drug-microcapsules lysate, indicating that cytotoxic activity was similar. The maximum reduction in cell activity for all three drugs and lysates was approximately 80%. For 5-FUdR and methotrexate, the steepest part of the dose response curve was from 1 μg/ml down to 0.01 μg/ml and for doxorubicin from 0.1 μg/ml, well within serum ranges seen *in vivo*. Control lysates of underivatised microcapsules showed no cytotoxic activity.

Example 8

This Example illustrates linkage of active compound, not directly to the microcapsule shell wall but *via* a degradable spacer or linker. This enables greater control of both the linking and release of the active compound.

Using the technology detailed in WO-A-9317713 for linking drugs to soluble carriers, naproxen has been linked to microcapsules using a lactic acid spacer. To a 10 mmol suspension of L-lactic acid in dimethylformamide, 20 mmol triethylamine and 10 mmol pentamethylbenzyl (PMB) chloride

were added. The mixture was heated until a solution was formed and then held at room temperature. Excess sodium carbonate was added after incubation of the solution overnight, and the precipitated ester, L-lactic acid-PMB, was collected, washed and dried.

To a solution containing 10 nmol naproxen, L-lactic acid-PMB and 4-dimethylaminopyridine, 11 mmol solution of dicyclohexylcarbodiimide was added. The reaction mixture was stirred at 25°C and the formation of the naproxen linker monitored. On completion of the reaction, the naproxen-L-lactic acid linker was collected, washed and dried.

The PMB protecting group was removed by the reaction of the naproxen linker with anisole and trifluoroacetic acid at room temperature for 2 minutes. Excess reagent was removed under vacuum and the residue was collected and washed. Acidification of the washed residue produced naproxen-L-lactic acid, which was extracted, washed and dried under vacuum at 50°C.

The naproxen-L-lactic acid was activated by its 1:1 reaction with carbodiimide, followed by the addition of 1 mmol N-hydroxysuccinimide. The active naproxen-L-lactic acid-NHS was added to HSA microcapsules at a 5:1 ratio in a borate buffer. The resultant product was collected and dried.

The dried naproxen microcapsules were formulated, resulting in a free-flowing powder, with a microcapsule mean size of 3.5 ± 0.6 μm. 90% of the mass of the microcapsules was between 2 and 5 μm.

Analysis of the product was carried out using Capillary Zonal Electrophoresis (Beckman, UK). This showed the presence of the drug on the microcapsules. The release of the drug using esterases and subsequent analysis of the released naproxen were carried out using an ASTED system linked to a Gilson HPLC (Anachem UK). The drug was shown to be intact and in its native form.

Example 9

The spray-drying production of the microcapsules allows control over many facets of the process and final characteristics of the microcapsules. The surface characteristics of the final microcapsules can be altered such that ligands for enzymes or receptors may be incorporated into the microcapsule shell. In this Example, the number of arginine residues is increased, and this enhancement was used to bind TPA.

Using the method of Examples 1 and 5, poly-arginine was added to the spray-drying feedstock. Using the same conditions as described in Example 1, microcapsules are produced. The microcapsules have a mean size of 3.31 ± 0.6 μm and 90 % of the mass is between 2 - 5 μm.

To 100 mg of microcapsules, a solution containing 250 μg TPA is added. The suspension is agitated for 2 hours after which the microcapsules are removed and briefly washed. The concentration of TPA remaining in the reaction solution is measured by RP-HPLC having reduced the peptide by incubation in 20 mM DTT at 37°C for 30 min in the presence of 8 M Urea. The analysis of the fragments is carried out using a gradient of 10-40% acetonitrile-water and 0.1% TFA over 60 minutes.

The TPA-microcapsules are analysed for the presence of TPA using the clot lysis assay. A fibrin clot is produced by combining fibrinogen, thrombin and the TPA microcapsules. Plasminogen is then added to the clot and a glass bead added to the surface to allow the assay end point, i.e. clot lysis, to be determined. The fall of the glass bead through the lysed clot shows that the TPA is both bound to the microcapsules and that it is still active.

In addition, the amount of TPA bound to the microcapsules is determined by using a modified fibrin assay. To a microtiter plate well, a thin agarose gel containing fibrinogen and thrombin is added. To the gel, 20 μl suspension of TPA-microcapsules and plasminogen are added. After 30 minutes, the plate is washed and the

reduction in the gel turbidity is determined using a microtiter plate reader at 340 nm. The concentration of TPA present on the microcapsule is determined using appropriate TPA standards. The results show that between 15 and 20 % TPA is bound to the microcapsules.

The TPA microcapsules can have utility as a deposit thrombolytic agent for administration at the time of angiography, similar to that proposed in WO-A-9408627 as a deposit echocontrast agent, the advantage being maintenance of a localised reservoir of TPA in the myocardium.

TABLE 4 AMINO ACID COMPOSITION OF NATIVE ALBUMIN AND MICROCAPSULES

Amino Acid	HSA (n = 5)	Soluble Microcapsules (n = 5)	Insoluble Microcapsules (n = 7)
Aspartic Acid	50.188 ± 2.73	57.99 ± 4.13	59.23 ± 4.81
Glutamic Acid	79.31 ± 5.44	80.47 ± 2.26	85.70 ± 4.04
Serine	23.21 ± 1.39	23.2 ± 1.23	20.65 ± 2.44
Glycine	14.54 ± 0.71	15.33 ± 0.38	14.93 ± 107
Histidine	17.34 ± 1.06	17.65 ± 1.01	16.42 ± 0.85
Threonine	26.99 ± 1.34	27.23 ± 1.56	28.39 ± 2.38
Alanine	63.83 ± 1.34	61.45 ± 0.96	62.69 ±1.86
Arginine	25.18 ± 0.95	24.39 ± 1.02	23.15 ± 1.55
Proline	28.36 ± 1.17	27.45 ± 2.03	25.20 ± 3.05
Tyrosine	18.37 ± 0.71	18.02 ± 0.42	16.13 ± 1.38
Valine	35.54 ± 1.49	35.07 ± 1.03	37.59 ± 4.53
Methionine	7.99 ± 0.5	7.68 ± 0.42	6.24 ± 1.16
Isoleucine	6.42 ± 0.54	6.64 ± 0.57	8.96 ± 1.78
Leucine	63.22 ± 3.48	62.13 ± 3.14	59.28 ± 4.38
Phenylalanine	32.00 ± 2.73	30.45 ± 2.13	30.48 ± 1.92
Lysine	56.185 ± 2.50	53.45 ± 1.62	51.68 ± 3.67

CLAIMS

1. A sterile powder comprising smooth, spherical microparticles, 0.1 to 50 μm in diameter, of cross-linked materials, the microparticles being hydrophilic and capable of reconstitution in water to give a mono-disperse suspension, and which additionally comprises a physiologically or diagnostically-active component linked directly or indirectly to microparticles *via* free functional groups thereon.

2. A powder according to claim 1, wherein the material is an amino-acid, a polyamino-acid or other polypeptide.

3. A powder according to claim 1 or claim 2, which incorporates an additional water-soluble material that facilitates enzymatic biodegradation.

4. A powder according to any preceding claim, wherein the active component is a drug, chemical spacer, a ligand for an enzyme or receptor, or an antibody.

5. A powder according to any preceding claim, obtainable from said material, which is water-soluble and has said groups, by (i) spray-drying and cross-linking, such that said groups are retained in free form, and (ii) linking the active component to the cross-linked material *via* said groups.

6. A powder according to claim 5, wherein step (i) comprises spray-drying a solution of the water-soluble material, and cross-linking the dried material with heat and in the presence of less than 4% moisture.

7. A powder according to any preceding claim, wherein the functional groups are amine, hydroxyl, carboxyl or sulfhydryl.

8. A powder according to claim 7, wherein the functional groups are amine, hydroxyl or carboxyl.

9. A powder according to any preceding claim, wherein the particles have a ratio of interquartile range to mean diameter of 0.2 to 0.5.

10. A powder according to claim 9, wherein 95% of the particles are smaller than 6 μm, and 80% of the particles are in the range of 1 to 6 μm.

11. A powder according to claim 9, wherein 90% of the particles are smaller than 20 μm, and less than 5% by volume are smaller than 6 μm.

12. A suspension of microparticles as defined in any preceding claim, suitable for parenteral administration.

13. A suspension according to claim 12, wherein the microparticles are according to claim 10, for intravenous administration.

14. A suspension according to claim 12, wherein the microparticles are according to claim 11, for intraarterial administration.